EX. D

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Deletion mapping of the *Aequorea victoria* green fluorescent protein *

(Autocatalysis; bacterial expression; bioluminescence; chromophore; *Cnidaria*; cyclization)

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SUMMARY

Aequorea victoria green fluorescent protein (GFP) is a promising fluorescent marker which is active in a diverse array of prokaryotic and eukaryotic organisms. A key feature underlying the versatility of GFP is its capacity to undergo heterocyclic chromophore formation by cyclization of a tripeptide present in its primary sequence and thereby acquiring fluorescent activity in a variety of intracellular environments. In order to define further the primary structure requirements for chromophore formation and fluorescence in GFP, a series of N- and C-terminal GFP deletion variant expression vectors were created using the polymerase chain reaction. Scanning spectrofluorometric analyses of crude soluble protein extracts derived from eleven GFP expression constructs revealed that amino acid (aa) residues 2–232, of a total of 238 aa in the native protein, were required for the characteristic emission and absorption spectra of native GFP. Heterocyclic chromophore formation was assayed by comparing the absorption spectrum of GFP deletion variants over the 300–500-nm range to the absorption spectra of full-length GFP and GFP deletion variants missing the chromophore substrate domain from the primary sequence. GFP deletion variants lacking fluorescent activity showed no evidence of heterocyclic ring structure formation when the soluble extracts of their bacterial expression hosts were studied at pH 7.9. These observations suggest that the primary structure requirements for the fluorescent activity of GFP are relatively extensive and are compatible with the view that much of the primary structure serves an autocatalytic function.

INTRODUCTION

The green-fluorescent protein (GFP) of *Aequorea victoria* is an accessory protein in a jellyfish bioluminescence pathway which relays the energy in the pulses of blue light emitted from the Ca^{2+} activation of aequorin as green luminescence (Johnson et al., 1963; Morin and Hastings, 1971). Interest in GFP has broadened since the demonstration that heterologous expression of the *gfp* cDNA in a variety of species produces a fluorescent protein (Chalfie et al., 1994; Inouye and Tsuji, 1994a; Wang and Hazelrigg, 1994; Sengupta et al., 1994). GFP's spectral characteristics, small size (238 aa), and acquisition of fluorescent activity in the absence of other

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Abbreviations: *A.*, *Aequorea*; A_{600}, absorbance at 600 nm; aa, amino acid(s); Ap, ampicillin; apoGFP, non-fluorescent apoprotein form of GFP; bp, base pair(s); cDNA, duplex DNA sequence representing a mRNA species; Δ, deletion; GFP, green fluorescent protein; *gfp*, gene encoding GFP (GenBank accession No. M62653) or a GFP deletion variant; GFPΔx-y, deletion variant of GFP where Δx-y identifies the range(s) of the aa deletion(s) from GFP; His_6, 6-aa polyhistidine domain; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase(s); LB, Luria-Bertani (medium); nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; [], denotes plasmid-carrier state.

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TABLE I
Oligos[a]

Oligo sequence	Name
5'-GACTGAATTCATGAGTAAAGGAGAAGA	GFP3
5'-GACTCTAGACTATTTGTATAGTTCATCC	GFP4
5'-GACTCTCGAGAGTAAAGGAGAAGAACTTTCACT	GP1
5'-GACTCTCGAGGGTGAAGGTGATGCAACATACGGA	GP2
5'-GACTCTCGAGAGATACCCAGATCATATGAAACAG	GP3
5'-GACTGGATCCTTATTTGTATAGTTCATCCATGCC	PG1
5'-GACTGGATCCTTAGGGATCTTTCGAAAGGGCAGATTG	PG2
5'-GACTGGATCCTTAAATGTTGTGTCTAATTTTGAAGTT	PG3
5'-GACTCTCGAGACTGGAGTTGTCCCAATTCTT	GPA
5'-GACTGGATCCTTACATGTGGTCTCTCTTTTCGTTGGG	PGX
5'-GACTGGATCCTTATGTTACAAACTCAAGAAGGACCAT	PGY
5'-GACTGGATCCTTAGCCATGTGTAATCCCAGCAGCTGT	PGZ

[a] The oligos were synthesized on an automated synthesizer (Applied Biosystems Model 392, Foster City, CA, USA) according to the manufacturer's instructions. Each oligo contains 10 nt of non-complementary sequence at the 5' end consisting of a 6-nt restriction site and a 4-nt clamp to assist in restriction digestion of the amplified sequences. GFP3 and GFP4 were used to amplify the complete *gfp* coding sequence for directional ligation into pMAL (New England Biolabs, Beverly MA; Guan et al., 1987) using *Eco*RI and *Xba*I restriction sites. The remaining oligos were used to amplify full length *gfp*, or in-frame fragments of *gfp*, for directional ligation into pET14b (Novagen, Madison, WI, USA, Rosenberg et al., 1987) using the *Xho*I and *Bam*HI restriction sites in this vector.

A. victoria proteins make it an attractive candidate as an in vivo marker for studies of promoter activity, cellular protein trafficking, gene transfer, and protein-protein interactions (Chalfie et al., 1994; Heim et al., 1994; Marshall et al., 1995).

The fluorescent properties of GFP arise from an internal chromophore formed from a post-translational modification of a ^{65}Ser-Tyr-Gly^{67} sequence in apoGFP yielding a heterocyclic ring structure (Cody et al., 1993). The ability of apoGFP to undergo these structural modifications in a variety of heterologous cellular environments implies that these modifications arise from interaction with a ubiquitous cellular co-factor and/or from an unknown autocatalytic mechanism (Chalfie et al., 1994). To date, only molecular oxygen has been identified as a possible co-factor for chromophore formation in GFP. (Heim et al., 1994; Inouye and Tsuji, 1994b).

The existing evidence that the GFP chromophore forms autocatalytically is largely based on the observation that expression of the *gfp* cDNA in cells from a variety of species produces a fluorescent protein. Additional evidence comes from the observations that purified recombinant GFP undergoes a reversible oxidation-reduction reaction in the presence of molecular oxygen to yield a fluorescent product (Inouye and Tsuji, 1994b) and that bacterial expression hosts for recombinant GFP grown anaerobically acquire fluorescence with pseudo-first order kinetics when oxygen and protein synthesis inhibitors are added to the culture (Heim et al., 1994). Whether the non-fluorescent GFP present in these experiments represented apoGFP, or reduced GFP already containing a cyclized chromophore, is presently unknown. If the formation of GFP is autocatalytic, the best evidence for this will come from isolation and analysis of the purified apoprotein.

Previous studies on the absorption spectra of denatured GFP (Ward et al., 1980; Bokman and Ward, 1981) and proteolytic fragments of denatured GFP (Cody et a., 1993) strongly suggest that a heterocyclic ring structure is preserved in both cases. However, the fluorescent activity of GFP is more readily extinguished than its absorbance over the 300–500-nm range as it is affected by high salt concentrations, temperatures greater than 70°C, great extremes of pH, and organic solvents (Ward et al., 1980). Interestingly, single aa substitutions located at a distance in the primary sequence from the chromophore region have been shown to affect the absorption spectrum of non-denatured GFP variants (Heim et al., 1994). Moreover, the same study reported that some single aa substitutions at Tyr^{66}, a component of the chromophore, would still result in a fluorescent GFP variant, albeit one with altered absorption and emission spectra. Another study reported that combinatorial mutagenesis of the chromophore region in GFP has produced single aa substitutions at Ser^{65}, a number of which were consistent with fluorescence and a red-shifted absorption spectrum (Delagrave et al., 1995).

Assessment of the fluorescent activity of GFP deletion variants was hoped to provide useful information to investigators seeking to generate fluorescent fusion proteins by defining a minimal domain necessary for fluorescence, irrespective of whether this is a requirement for a minimal enzyme or a minimal substrate. Also, since the fluorescence of GFP depends not only on the formation

TABLE II

Fluorescence in expression vector constructs[a]

Primer combination[b]	Expression vector[c]	Number of clones evaluated[d]	Number of fluorescent clones[e]
GFP3+GFP4	pMAL/GFP	44	42
GP1+PG1	pET14b/GFP	2	2
GP1+PG2	pET14b/GFPΔ213–238	2	0
GP1+PG3	pET14b/GFPΔ173–238	2	0
GP2+PG1	pET14b/GFPΔ2–32	2	0
GP2+PG2	pET14b/GFPΔ2–32,213–238	2	0
GP2+PG3	pET14b/GFPΔ2–32,173–238	2	0
GP3+PG2	pET14b/GFPΔ2–72,213–238	2	0
GP3+PG3	pET14b/GFPΔ2–72,173–238	2	0
GPA+PG1	pET14b/GFPΔ2–8	2	0
GP1+PGX	pET14b/GFPΔ219–238	4	0
GP1+PGY	pET14b/GFPΔ226–238	4	0
GP1+PGZ	pET14b/GFPΔ233–238	4	3

[a] Fluorescence was scored by direct examination of bacterial colonies illuminated with a 365 nm ultraviolet light source. GFP production was induced by re-streaking isolated colonies onto fresh culture plates bearing discs of 80 mM IPTG-saturated Whatman filter paper.

[b] Deletion mutant *gfp* cDNA's were created by PCR amplification of a cloned circular template, TU#65 (Chalfie et al., 1994). The conditions for amplification were 100 ng of circular template/4 mM $MgCl_2$/5 units Amplitaq (Perkin-Elmer, Branchburg, NJ, USA) with manufacturer's buffer/0.2 mM deoxynucleotide triphosphates each, and a thermal cycler program as follows: (95°C × 5 min, 37° × 5 min, 72°C × 5 min) for 5 cycles followed by (95°C × 45 s, 45°C × 2 min, 72°C × 3 min) for 20 cycles. Primer oligo sequences and their names are in Table I.

[c] The PCR products were purified over mini-columns (Promega, Madison, WI, USA) and digested with appropriate restriction enzymes (*Eco*RI and *Xba*I were used for the product of the GFP3 and GFP4 primer combination. *Xho*I and *Bam*HI were used for all other combinations.) Expression vector backbones and *gfp* inserts were gel-purified by electrophoresis (1% agarose in 1 × TAE buffer (Tris·acetate 0.04 M/EDTA 0.001 M), electrophoresis at 3 V/cm, 22°C, 90 min) in low-gelling temperature agarose (GIBCO/Life Technologies, Gaithersburg, MD, USA). Bands were excised and in-gel ligation was conducted by standard methods (Sambrook et al., 1989). The ligation reaction was used to transform the XL-1, Blue bacterial strain by electroporation (14 kV/cm, 25 μF, 200 Ω, 4°C). Correct colonies were identified by restriction digest mapping. pET14b-derived (Novagen, Madison, WI, USA; Rosenberg et al., 1987) expression vectors were transferred to a compatible expression host, BL21(DE3)[pLysS] (Studier and Moffatt, 1986) to study the behavior of the GFP deletion variants. The pET14 b-derived vectors produced GFP and its deletion variants as His_6-tagged N-terminal fusion proteins. Apart from affecting the electrophoretic mobility of GFP, these 2.5-kDa regions appear not to alter the activity of GFP (Inouye and Tsuji, 1994a). The pMAL-derived vector produced full-length GFP as a maltose-binding protein fusion (Guan et al., 1987) of 69.5-kDa. The production of the fluorescent maltose-binding protein/GFP fusion could be assessed directly in XI-1, Blue bacteria after IPTG induction.

[d] For the pET14b-derived expression constructs, independent clones of each construct were restriction-mapped to verify the presence of a correct insert. Correctly mapping clones were used to transform the BL21(DE3)[pLysS] expression host for fluorescence screening. For the pMAL-derived expression construct, six of the original XL-1, Blue colonies were restriction-mapped and all found to contain the correct insert. These six colonies, and a further 38 unmapped colonies, were re-streaked onto fresh plates and screened for fluorescence after IPTG induction.

[e] Colonies were scored for fluorescence 8 to 12 h after IPTG induction although the colonies which proved to be fluorescent could usually be scored accurately 2 to 4 h after induction and growth at 37°C.

of a chromophore but also on the structural relationship of the chromophore to the rest of GFP (Bokman and Ward, 1981), we reasoned that determination of the absorption spectra of GFP deletion variants might identify a deletion which extinguished fluorescence but which preserved an absorption spectrum suggestive of heterocyclic chromophore formation thereby defining a minimal structural requirement for modification of the apoprotein.

EXPERIMENTAL AND DISCUSSION

(a) Construction of the expression vectors

All the expression constructs described in this paper were derived from TU#65 (Chalfie et al., 1994) by PCR amplification (Saiki et al., 1988) of its *gfp* cDNA coding region. TU#65 is a pGFP10.1 derivative. The latter is reported to have a single base pair change from the published sequence, $Gln^{80} \rightarrow Arg$ (Chalfie et al., 1994). The complementary regions of the primers (Table I) were designed with reference to the published sequence for the *gfp10* cDNA (Prasher et al., 1992; GenBank accession No. M62653). The pMAL/GFP vector contains the first ATG codon from *gfp10* although there is no evidence it functions as a translation start signal in this context. The remaining expression constructs have an in-frame deletion of this codon and rely on the translational start signal supplied by the vector. C-terminal deletions were constructed by adding an in-frame TAA stop codon to the antisense primer. All the PCR-generated inserts were cloned into the expression vector backbones by directional ligation.

The observed rate at which the PCR conditions pro-

duced non-fluorescent XL-1, Blue[pMAL/GFP] colonies (Table II) was used to estimate the likelihood that an individual bacterial expression vector clone would fail to fluoresce due to random amplification errors. The pMAL/GFP construct was easier to screen for this purpose because it will express in many ordinary bacterial host strains which can be made highly electrocompetent. Two out of 44 screened colonies of XL-1, Blue-[pMAL/GFP] failed to fluoresce upon ultraviolet irradiation. The observed rate of non-fluorescence due to presumed random error was 4.5%, which suggested that the fluorescent activity of the GFP deletion variants produced by individual clones of pET14b expression constructs was likely to be a true representation of the effect of the designed deletions. The observed concordance of fluorescent activity among the individual clones of each pET14b construct was quite high with only one exception. A single pET14b/GFPΔ233–238 clone scored as non-fluorescent when the other three replicates of this construct scored as fluorescent.

The individual pairs of primers produced inserts with a family of nested in-frame N- and C-terminal deletions (Fig. 1). After the initial series of deletion variants (pET14b/GFP, pET14b/GFPΔ213–38, pET14b/GFPΔ173–238, pET14b/GFPΔ2–32, pET14b/GFPΔ2–32, 213–238, pET14b/GFPΔ2–32, 173–238, pET14b/GFPΔ2–72, 213–238, and pET14b/GFPΔ2–72, 173–238) showed that only the full-length cDNA produced a fluorescent protein, a series of finer deletions were generated

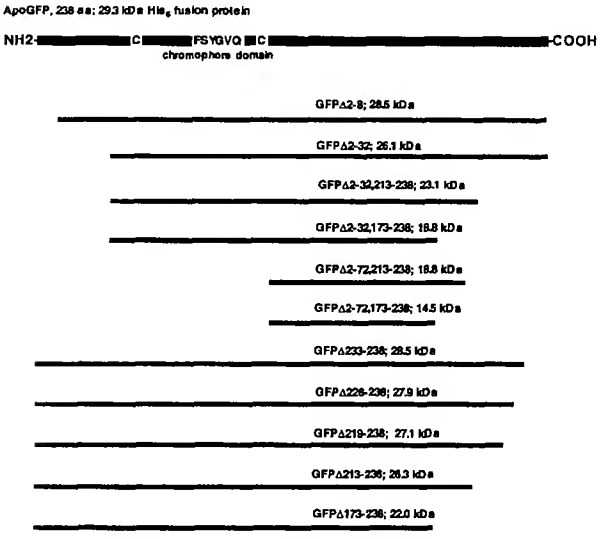

Fig. 1. GFP deletion variant schematic. The substrate tripeptide, Ser-Tyr-Gly, and its flanking aa found in the chromophore-containing proteolytic fragment (Cody et al., 1993) are indicated in their positions between Cys^{48} and Cys^{70} in apoGFP. The His_6 domains are not shown. The predicted sizes are calculated for the His_6-GFP fusions. The deletion variants GFPΔ2–72, 173–238 and GFPΔ2–72, 213–238 do not contain a chromophore domain.

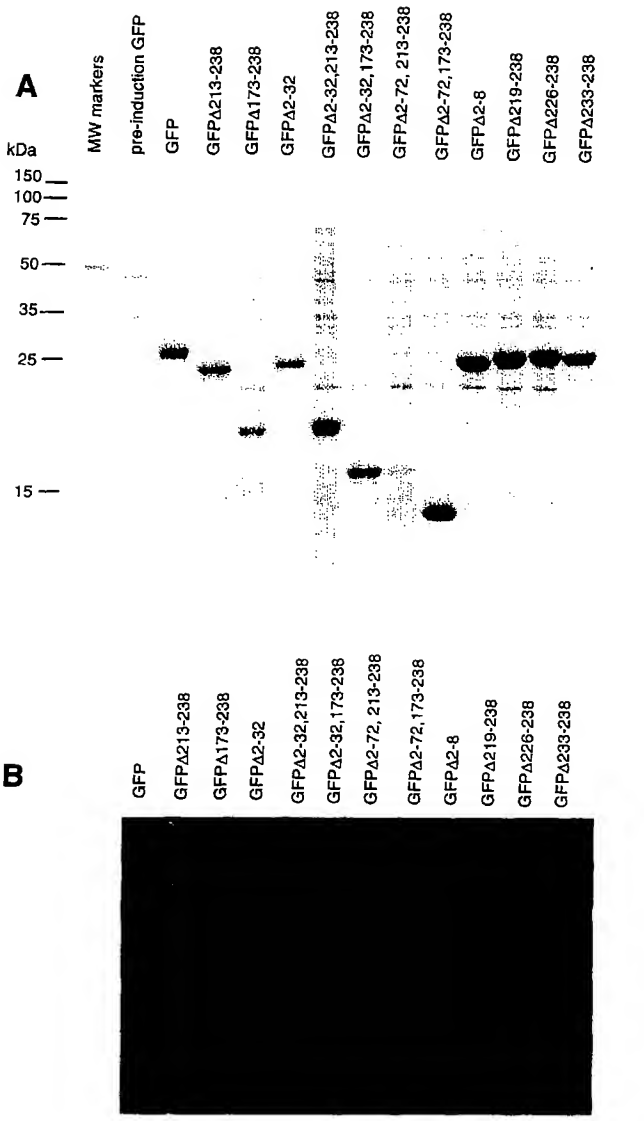

Fig. 2. 0.1% SDS-4–12% PAGE of His_6-GFP deletion variant fusion proteins. **A:** Cultures of BL21(DE3)[pLysS] were grown to an A_{600} of 0.5 to 0.7 at 37°C in LB media containing 100 μg Ap/mL before induction with 0.4 mM IPTG and further growth for 4 h. 1.0 mL of pre-induction culture medium and 0.5 mL of post-induction culture medium were centrifuged and re-suspended in 50 and 100 μL, respectively, of reducing, denaturing gel loading buffer (50 mM Tris (pH 7.0)/10 mM β-mercaptoethanol/2% SDS/0.1% bromophenol blue). Samples were heated for 30 min at 94°C before 20 μL of each was analyzed by Laemmli 4–12% PAGE, at a constant current setting of 35 mA for 5.5 h at 22°C in running buffer containing 25 mM Tris (pH 8.3)/192 mM glycine/0.1% SDS. Coomassie staining was performed by a standard technique (Sambrook et al., 1989). **B:** PAGE of the same post-induction specimens under native conditions. Treatments with SDS, β-mercaptoethanol, and heat were omitted. The samples were analyzed by 4–12% PAGE for 6 h at a constant voltage setting of 500 volts and a temperature of 4°C without SDS. The running buffer consisted of Tris 25 mM (pH 8.3)/glycine 192 mM. The gel was photographed through a Wratten 61 filter with 365 nm ultraviolet transillumination.

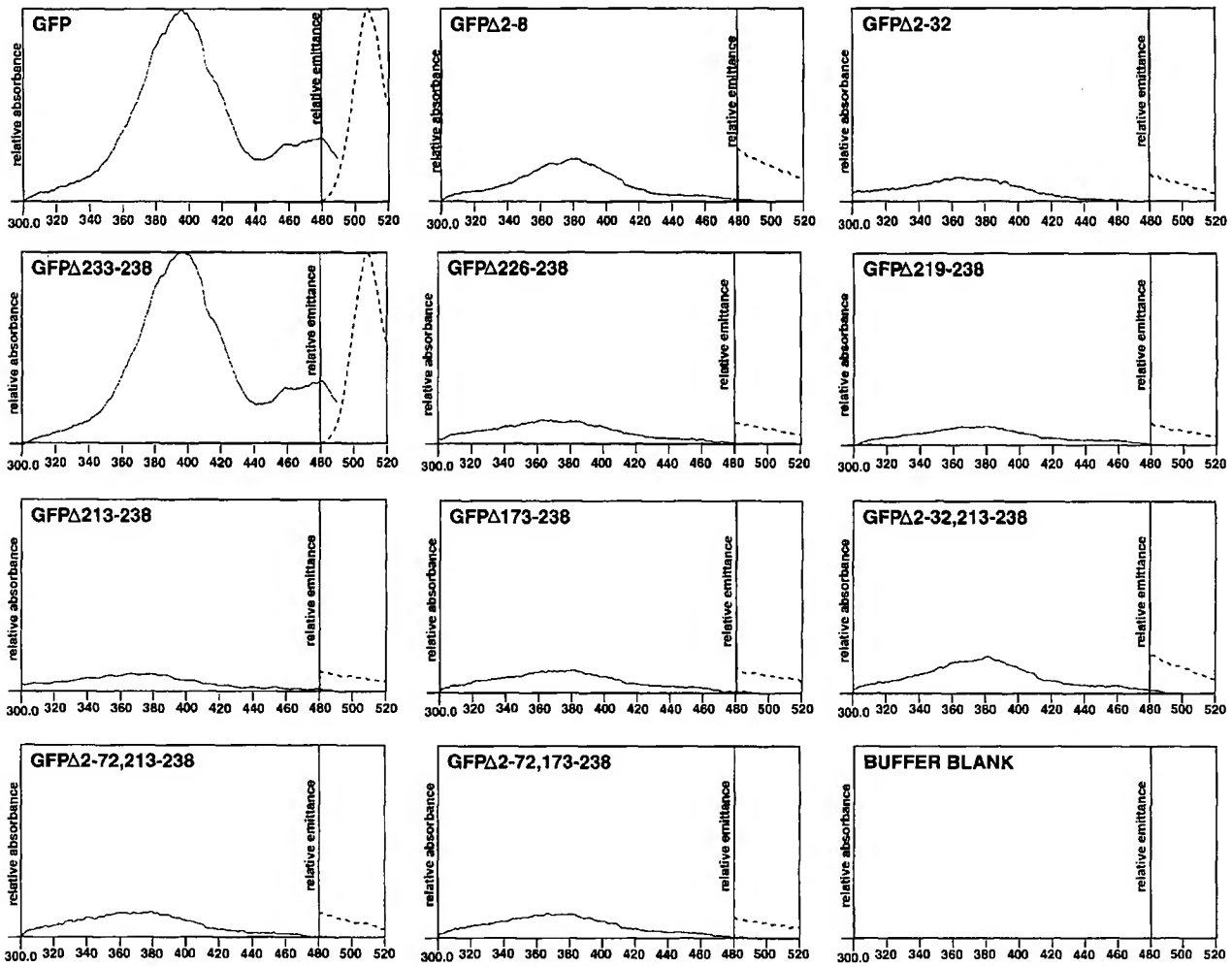

Fig. 3. Absorption (solid lines) and emission (dashed lines) spectra of His_6-GFP deletion variant fusion proteins. Bacterial cultures (200 mL of YT medium containing 100 μg Ap/mL) were grown at 37°C to an A_{600} of 0.5 to 0.7 before induction with 0.4 mM IPTG and an additional 3 h of post-induction growth. 150 μL of the post-induction culture media were resuspended in SDS–PAGE loading buffer and heated prior to PAGE to assess the adequacy of IPTG induction. The remainders of the cultures were centrifuged at 8000 × *g* and the solids resuspended in 10 mL of a buffer containing 5 mM imidazole/0.5 M NaCl/20 mM Tris·HCl (pH 7.9). The suspensions were subjected to three freeze-thaw cycles and 10 min of centrifugation at 39 000 × *g*. The fluorescent samples were inspected for retention of bright fluorescence in the supernatant. All samples were otherwise handled identically. Absorption and emission spectra of the supernatants were obtained with a Perkin-Elmer Model LS50 luminescent spectrometer. Absorbance was measured over the range of 300–500 nm at a scanning speed of 100 nm/min and a 5 nm bandwidth. Emittance was measured over the 480–520-nm range during 395 nm irradiation. Plotted values have been normalized to those observed for the GFP sample in this experiment.

(pET14b/GFPΔ2–8, pET14b/GFPΔ219–238, pET14b/GFPΔ226–238, and pET14b/GFPΔ233–238). The results of the latter series of deletions showed that GFP fluorescence would be retained in the face of a C-terminal deletion as large as 6 aa residues. The next largest C-terminal deletion variant lacked 13 aa and was not fluorescent. The smallest N-terminal deletion presented in this study, contained in GFPΔ2–8, eliminated fluorescent activity from the protein. The two clones, pET14b/GFPΔ2–72, 213–238 and pET14b/GFPΔ2–72, 173–238, lacked the aa which contribute to formation of the chromophore and were expected to serve as negative controls.

(b) Electrophoretic mobility and fluorescent activity

PAGE of the polyhistidine-GFP deletion variant fusion proteins (Fig. 2A) showed that the migration of the induced bands under denaturing conditions closely matched that calculated for their predicted aa compositions and that which has been reported for purified native GFP (Prendergast et al., 1978). The production levels of the induced proteins was approximately the same among the GFP deletion variants except for pET14b/GFPΔ2–72, 213–238 which repeatedly failed to produce as prominent an induced band as the other constructs. Under native conditions (Fig. 2B),

GFPΔ233–238 migrates more slowly than full length GFP possibly owing to the absence of two acidic aa, Asp^{234} and Glu^{235}, removed by the deletion.

(c) Scanning spectrofluorometry

Crude, soluble extracts of the post-induction BL21(DE3)[pLysS] cultures were studied by scanning spectrofluorometer (Fig. 3). The absorption and emission spectra of GFPΔ233–238 were essentially identical to that of full-length GFP observed in this study and reported in the literature (Ward et al., 1980; Chalfie et al., 1994). Among the non-fluorescent deletion variants, the absorption spectra did not differ between those constructs coding for the chromophore domain and those that did not code for it. This suggests that the minimum domain required for cyclization of the chromophore region could not be resolved from the minimum domain required for fluorescence in these experiments.

(d) Conclusions

Although a finer map may slightly revise it, a conservative estimate of the size of the minimal domain required for fluorescence in GFP extends from aa 2 to 232 in the native protein. Evidence of formation of a cyclized chromophore domain, which has been detectable by its absorption spectrum in the 300–500-nm range in non-fluorescent, denatured GFP (Ward et al., 1980; Bokman and Ward, 1981) and in non-fluorescent, proteolytic fragments of GFP (Cody et al., 1993) was not found among any of the non-fluorescent GFP deletion variants. Accordingly, we propose that residues 2–232 are also a provisional estimate of the minimal domain required for cyclization of the chromophore-forming tripeptide in apoGFP. More detailed studies of affinity-purified (Porath and Olin, 1983) GFP deletion variants, especially the pH response of their absorption spectra, will help confirm this proposition.

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